Original Article

Schistosoma mansoni soluble egg antigens enhance HCV replication in mammalian cells

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Abstract

Background: This work demonstrates successful propagation of HCV in HepG2 and human blood cells as well as viral shedding into their culture media. The influence of *Schistosoma mansoni* crude soluble egg antigens (SEA) on the rate of viral propagation in both mammalian cells was also monitored.

Methodology: HepG2 cells were inoculated with HCV viremic human sera and some wells were exposed to HCV infection in presence of SEA. Cells were harvested for RT-PCR and Western blotting analysis. HepG2 media was collected for HCV ELISA. Blood samples from HCV-infected humans were cultured in the presence and absence of SEA. Media were collected at different time points post culturing and subjected to HCV ELISA.

Results: The ELISA concentration of HCV antigens were generally higher in media of infected HepG2 cells compared to media of control cells at all time intervals post infection. Western blots showed reactivity to immunogenic peptides of different molecular weights in lysate of infected HepG2 cells that were not evidenced in uninfected cells. In presence of SEA, RT-PCR results revealed earlier detection of viral RNA in infected HepG2 cells compared to in absence of such bilharzial antigen. Also, ELISA results revealed higher levels of detected HCV antigens in media of both infected HepG2 and blood cells cocultured with *S. mansoni* SEA compared to that of cultured infected cells in absence of the parasite antigens.

Conclusion: HepG2 cells as well as whole blood cultures maintain HCV replication. Furthermore, SEA has the potential to enhance HCV propagation.

Key words: *Schistosoma mansoni* SEA, HCV replication, mammalian cells

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Introduction

Schistosomiasis is a chronic parasitic disease in tropical and subtropical regions and is associated with a variety of clinical syndromes that may lead to severe morbidity. Due to control programs over the last decade, a decline in the prevalence of human schistosomiasis in Egypt has been reported [1]; however, the disease is still endemic in many foci [2]. The colonic form of the disease [3] exists because of the presence of the parasite *Schistosoma Mansoni* (*S. mansoni*) and its intermediate snail host *Biomphalaria alexandrina* [4]. In *S. mansoni*-infected humans or animals, morbidity results from granuloma formation due to cell-mediated immune responses against deposited parasite ova (source of SEA) in the hepatic portal tracts that may progress to irreversible fibrosis and consequently severe portal hypertension [5].

Egypt has the highest HCV countrywide prevalence ranging from 6% to more than 40% among regions and demographic groups [6-7] with frequently reported coinfection with schistosomiasis [8-9]. Transmission of HCV among schistosomiasis-infected humans could have happened through multiple injections of humans with poorly sterilized syringes during the early anti-schistosomiasis mass treatment campaign [10-11]. Coinfected patients are characterized by higher HCV RNA titers, histological

activity, incidence of cirrhosis and hepatocellular carcinoma as well as higher mortality rates than patients with single infections [12-13].

The mouse model is susceptible to *S. mansoni* infection [14] but not to HCV; thus it is not adequate to study schistosomiasis/HCV coinfection in real time. Although the chimpanzee is proven to be susceptible to HCV infection [15] and can be used in studying coinfection, difficulties in maintaining representative numbers of animals to conduct all possible coinfection experiments make it an inappropriate model for such purposes. These challenges reflect the urgent need to develop a practical *in vitro* system that enables investigating the impact of coinfection on the progress of liver disorders.

The first attempts to establish *in vitro* HCV replication systems were conducted by infecting primary hepatocyte cultures [16-18]. Due to restricted availability of primary hepatocytes, the immortalized human hepatoma cell line HepG2 was later successfully used to host HCV replication *in vitro* [19-21]. In addition, several reports demonstrated replication and assembly of HCV-genotype 4 in peripheral blood mononuclear cells (PBMCs) from Egyptian infected patients [22-23]. Herein we confirmed successful HCV propagation in both human hepatoblastoma and blood cells. We also monitored the influence of *S. mansoni*-SEA on the viral propagation rate.

Materials and methods

Human sera

Sera were collected from HCV-infected patients who were examined at the medical unit at the National Research Center. The diagnosis of these patients was based on biochemical testing of liver enzymes and serological testing for the presence of anti-HCV antibodies using a recombinant HCV antigen-based test (Axium HCV rapid test, Florida, USA). HCV Infection of the positive individuals in the above-mentioned tests was further confirmed by RT-PCR. Sera from humans with no history of liver complications or disorders that were negative in all the above-mentioned tests were included as negative controls.

Cell line

The Caucasian male Homo sapiens hepatoblastoma cell line (HepG2) used as host cells for HCV replication was a kind gift from Prof. Ralf Bartenschlager, Director of the Institute of Molecular Virology, University of Heidelberg, Germany.

Detection of HCV-RNA in patients' sera by RT-PCR

RNA was extracted from human sera using the QIAmp viral RNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted into 2 ml nuclease-free Eppendorf tubes, divided into 10 µl aliquots and stored at -80°C. The extracted RNA was subjected for RT-PCR using a one-step RT-PCR kit (QIAGEN) that enables first strand cDNA synthesis and PCR amplification in one reaction mix. Forward (F; 5' GCA GAA AGC GTC TAG CCA TGG CGT 3') and reverse (R; 5' CTC GCA AGC ACC CTA TCA GGC AGT 3') primers (Operon Biotechnologies, Germany) were designed to specifically anneal to conserved regions within the HCV-5'UTR [24] and enable amplification of the 243 bp viral fragment.

The RT-PCR reaction mixture was performed in a final volume of 50 µl in a 0.2 ml nuclease-free Eppendorf tube containing 10 µl RNA template, 10 µl of 5X one-step RT-PCR buffer, 100 pmol of both F & R primers, 2 µl of dNTP's mixture, 2 µl RT-PCR enzyme mix and the volume was completed to 50 µl by nuclease-free water. The PCR tubes were inserted into the heating block of a DNA thermal cycler (Biometra, Goettingen, Germany) and the heating lid was enabled. The RT-PCR was started with first strand, cDNA, synthesis at 50°C for 30 minutes followed by hot start polymerase activation at 95oC for 15 minutes. The PCR amplification program included 36 cycles each consisting of 3 stages for template denaturation at 94°C for 30 seconds, primers annealing at 58°C for 30 seconds, and nucleotides addition (extension) at 72°C for 1 minute. The last cycle was linked to a final extension step at 72°C for 10 minutes followed by cooling at 4°C until the tubes were removed from the machine.

The PCR products were mixed with a 6X gel loading dye (SibEnzyme, Russia) to reach a final concentration of 1X before being loaded on to 1.5% agarose gel containing ethidium bromide (EB). An appropriately diluted stepladder (SibEnzyme) was also loaded on the same gel. The lid of the gel tank (Greiner bio-one, Germany) was closed; cables were connected to the power supply (Bio-Rad) and a voltage of 1-5 V/cm was applied for 30 to 45 minutes. The PCR products resolved by EB stain were visualized in comparison to the DNA marker on a UV transilluminator of a gel documentation system (Biometra, Goettingen, Germany).

Culturing HepG2 cells

Aliqouts of HepG2 cells were taken out of the liquid nitrogen tank and allowed to thaw on ice. Cells were maintained in 75 cm^2 culture flasks (Greiner) containing RPMI medium (Biowhittaker, Combrex, Belgium) supplemented with 0.45% glucose, 1% L-glutamine, 10% fetal bovine serum (FBS; Biowest, France) and 1% antibiotic-antimycotic mixture (GIBCO-BRL, New York, USA) at 37ºC with 5% CO^2. The medium was renewed every three days. To avoid overgrowth, cells were subcultured every three to five days. Briefly, the medium was discarded and an overlayer of trypsin-EDTA (Biowest) was added to the adherent cell layer to remove any leftover traces of trypsin inhibitors. After discarding the medium, the cell sheet was overlaid with 2 ml fresh trypsin-EDTA for 0.5 to 2 minutes at 37ºC and cell detachment was carefully observed under an inverted microscope. To avoid a harmful proteolytic effect of trypsin on the detached cells, complete medium containing excess FBS was added. Cells were spun down at 500 g for 2 minutes and resuspended in 1 ml of complete medium; their exact count and viability were microscopically checked by mixing equal volumes of the cells and trypan blue followed by loading a drop on a hemocytometer (Sigma, Deisenhofen, Germany). A total of 6 x 10^5 of the resuspended cells were used to prepare fresh cultures in flasks or 12-well plates (Greiner) and incubated at 37^{o}C under 5% CO_2 to be used later in infection experiments.

Infection of HepG2 cells with HCV

Infection of HepG2 cells was performed according to protocols described previously with some modifications [19,25]. In 12-well plates, cells were grown for 24 hours to semi-confluence in complete medium, washed three times with FBS-free medium then inoculated with sterile filtered HCV-infected serum through a 0.22 µm syringe filter to a final concentration of 10%. To study any possible influence for bilharzial antigens on HCV replication, some wells were exposed to HCV infection in the prsence of SEA (10 µg/ml; Theodore Bilharz Reasearch institute, Giza, Egypt). Plates were maintined in complete media for two weeks with daily microscopic observation. Cell harvesting was done by removing the media and scraping the cell monolayer either in 1 ml of the RNA extraction reagent (BIOZOL; BioFlux, Japan) for RT-PCR or 200 µl PBS for Western blotting analysis. Infection success and virus exocytosis in the collected media were checked by ELISA, Western blotting, and RT-PCR.

Peripheral whole blood culturing

Human whole blood culturing was conducted according to Itoh *et al*. [26] and Mutapi *et al*. [27] with some modifications. Briefly, freshly withdrawn venous blood samples from HCV-infected or healthy humans on EDTA were immediately diluted 1:4 in complete medium and distributed 1 ml/well in 12-well plates. To study any possible influence for bilharzial antigens on HCV replication, *S. mansoni* SEA was added (10 µg/well) to some of the infected blood containing wells. To monitor the capacity of cultured blood to support HCV replication, media were collected at days 1, 2, 3 and 6 post culturing and subjected to ELISA detection of viral antigens.

Quantitative detection of viral antigens in media of infected cells by ELISA

The assay was performed according to Bahgat *et al*. [28] with minor modifications. Briefly, 96-well polyvinyl microtiter ELISA plates (ALTO, Italy) were coated (100 µl/well) with the diluted media of infected HepG2 or blood cells either in absence or presence of SEA at different time intervals post infection in coating buffer (4.53 ml 1M sodium bicarbonate, 1.82 ml 1M sodium carbonate, 93.65 ml ddH2O, pH 9.6). Wells coated by media of uninfected cells were included in the same plate as means of negative controls. Plates were incubated at 37o C for 3 hours then washed 3 times with PBS-0.05%Tween20. Uncoated spaces on plate wells were blocked against non specific binding by being incubated with PBS-0.05% Tween-5%FBS (PBST-FBS; 200 µl/well) at 37°C for 2 hours. After 3 washes, wells were incubated with 100 µl of the first antibody which was either anti-HCV antibody positive human sera or mouse anti-sera raised against a DNA mammalian expression construct encoding HCV-NS3 protease [29] or mouse anti-HCV core monoclonal antibody (Virogen, Watertown, MA, USA) at 37°C for 2 hours. Both human sera or mice anti-NS3 antibodies were used at a dilution of 1:100 while the mouse anti-core-antibody was used at 1:2000 in PBST-FBS. Plates were washed 3 times and wells were loaded with anti-host-IgG peroxidase (Bio-Rad Laboratories, Munich, Germany; 1:10000, 100 µl/well) at 37°C for 2 hours followed by 3 washes. For visualization of the immune reaction, a volume of 100 µl/well of O-phenylenediamine substrate diluted in H_2O_2 containing substrate buffer

(49.6 ml 0.1M citric acid anhydrous, 50 ml 0.2M dibasic sodium phosphate, pH 5.00) was applied and plates were left for 10 minutes at room temperature until color development. Stopping solution (2M H2SO4, 50 µl/well) was used to terminate the enzymatic reaction and the changes in optical densities (OD) were recorded at λmax 492 nm using a multi-well plate reader (Tecan, Sunrise, Austria).

Detection of viral antigens in lysates of infected HepG2 cells by Western blotting

Following SDS-PAGE [30], proteins were electrophoretically transferred [31] from the gel to a nitrocellulose sheet (Schleicher & Schull, Dassel, FRG) at 250 mA overnight at 4°C in transfer buffer. On the next day, membranes were cut into individual strips of 0.3 mm width each. Strips were washed 3 times with PBS-0.3%Tween20 for 5 minutes and blocked against non specific binding by being soaked in PBS-0.3%Tween20 containing 1% bovine serum albumin for one hour at room temperature while shaking. Strips were washed 3 times as mentioned above and incubated with anti-HCV-antibody positive human sera (1:100 in PBS-0.3%Tween20) for 2 hours at room temperature while shaking. After 3 washes, strips were incubated for 2 hours at room temperature with diluted peroxidase labeled sec. antibodies (Bio-Rad; 1:1000 in PBS-0.3%Tween 20). Visualization of immune complexes on the nitrocellulose membrane was done by incubating the strips with the peroxidase specific precipitating substrate (3,3`diaminobenzedine tetrahydrochloride (50 mg, Sigma) in PBS (100 ml; 0.01M, pH 7.4) containing 10 µl of 30% H2O2; Sigma).

RNA extraction from infected HepG2 cells and HCV detection by RT-PCR

RNA was extracted from HCV-infected control HepG2 cells as well as their media for 6 successive days starting from day 1 post infection using the BIOZOL reagent according to the manufacturer's instructions. Briefly, the monolayer of the cells was scraped in a 1 ml BIOZOL reagent/well then collected in a 2 ml nuclease-free Eppendorf tube, vortex shaken for 15 seconds, and chilled on ice. After 15 minutes, 200 µl of chloroform were added to each tube, the mixture was vortex shaken for 15 seconds, and then incubated for 15 minutes at room temperature. Tubes were centrifuged at 13,000 g for 15 minutes at room temperature; the upper phases were carefully transferred to fresh 2 ml nuclease-free Eppendorf tubes containing equal volumes of cold isopropanol followed by gentle mixing by 5 inversions and overnight incubation at -20°C. On the next day, the tubes were centrifuged at 13,000 g for 15 minutes at 4° C, supernatants were discarded and RNA pellets were washed with 80% ethanol in nuclease-free water. After centrifugation at 13,000 g for 5 minutes, ethanol was discarded and RNA pellets were air dried from any remaining alcohol droplets before being resuspended in 60 µl nuclease-free water and then divided into 10 µl aliquots and stored at -80°C. The extracted RNA was then subjected to RT-PCR detection of HCV using a one-step RT-PCR kit as mentioned previously. Electrophoresis and visualization of the PCR products was performed as mentioned above.

Results

Confirming viral infection of human sera by RT-PCR

The RNA extracted from the sera of infected humans who were all anti-HCV antibody positive was exposed to RT-PCR detection of HCV. The RT-PCR revealed 243 bp amplification products while RNA from anti-HCV antibody negative sera was PCR negative reflecting the specificity of the primers for detecting infection (data not shown). The RT-PCR positive samples with the highest band intensities were used in infecting the HepG2 cells.

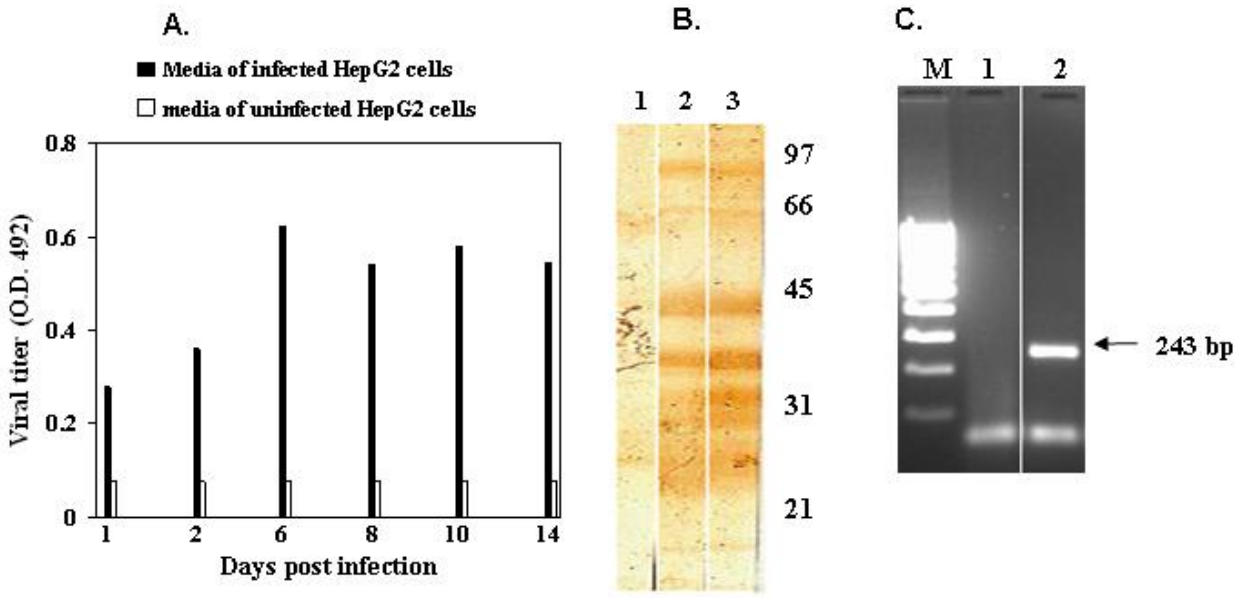
Evidences for HCV propagation in infected HepG2 cells

Using anti-HCV-antibody positive sera, the recorded OD corresponding to the concentration of HCV antigens was generally higher in media of infected HepG2 cells compared to media of control cells at all time intervals post infection (Figure 1A). Also, in the period between the first and sixth days post infection there was a gradual increase in the detected HCV antigens.

In Western blots (Figure 1B), anti-HCV-antibodies positive human sera reacted to immunogenic peptides at 105, 76, 43, 34, 30, 27 and 22 kDa in lysate of infected HepG2 cells at weeks 1 and 2 post infection (strips 2 and 3). The reaction was not evidenced in the lysate of uninfected cells (strip 1).

Starting from 4 days post infection, the extracted RNA from HepG2 cells gave a 243 bp amplification product by RT-PCR that was not evidenced in RNA from previous time points or in uninfected cells (Figure 1C).

Figure 1. Evidence for HCV propagation in infected HepG2 cells



A. Using anti-HCV-antibody positive sera, the recorded OD corresponding to the concentration of HCV antigens was generally higher in media of infected cells compared to media of control cells at all time intervals post infection. Between the 1st and 6th day post infection there was a gradual increase in the detected HCV antigens confirming virus shedding from the infected cells. B. In Western blots, anti-HCV-antibodies positive human sera reacted to immunogenic peptides at 105, 76, 43, 34, 30, 27 and 22 kDa in lysates of infected cells at weeks 1 and 2 post infection (strips 2 & 3), which was not evidenced in lysate of uninfected cells (strip 1). C. Starting from 4 days post infection, extracted RNA from HepG2 cells gave an 243 bp RT-PCR product (lane 2) that was not evidenced in RNA from previous time points or uninfected cells (lane 1).

Influence of S. mansoni *SEA existence on HCV propagation in HepG2 cells*

Using the anti-HCV-core monoclonal antibody in ELISA, the recorded core titers at all time points post infection were generally higher in media of infected cells than those in control cells (Figure 2). Addition of *S. mansoni* SEA to the growth medium of infected HepG2 caused increases in the quantified core titers in the media at different time points post infection when compared to media of infected cells in absence of such parasite antigens (Figure 2).

Influence of S. mansoni *SEA existence on HCV propagation in cultured human blood cells*

Using mouse anti-HCV-protease antibodies in ELISA detected higher NS3 titers in media of human blood infected cells compared to that of control cells (Figure 3). The quantified NS3 titers were higher in media of cultured blood cells in presence of SEA than in its absence, supporting the above-mentioned enhancing effect of SEA on HCV propagation observed in HepG2.

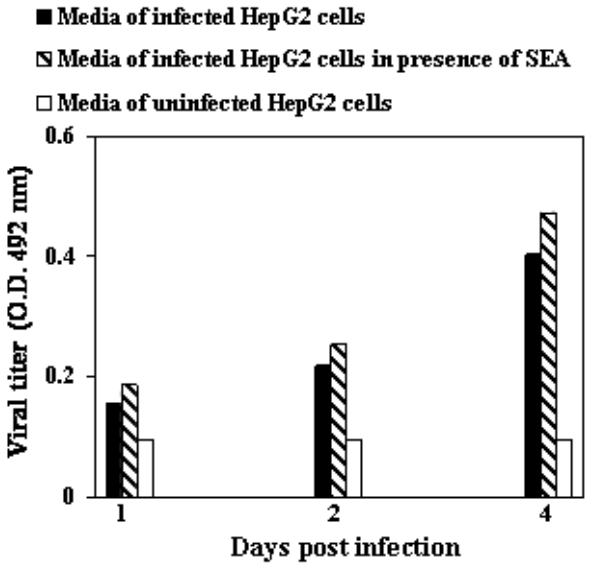
Discussion

The molecular weight of the amplified viral fragment (243 bp) by RT-PCR agreed with the annealing position of the used primers [24] and the absence of amplification products from control human sera reflects the specificity of the used primers in diagnosing HCV infection.

Using anti-HCV-antibody positive sera, the recorded OD corresponding to the concentration of HCV antigens was generally higher in media of infected cells compared to media of control cells at all time intervals post infection and there was a gradual increase in the detected HCV antigens during the six days post infection confirming virus shedding from the infected cells. The susceptibility of HepG2 cells to HCV infection and its support for viral replication agree with previous reports [19,21,25]. The sensitivity of human anti-HCV-antibody positive sera to detect HCV antigens in infected HepG2 agrees with the observations of El-Awady *et al*. [21].

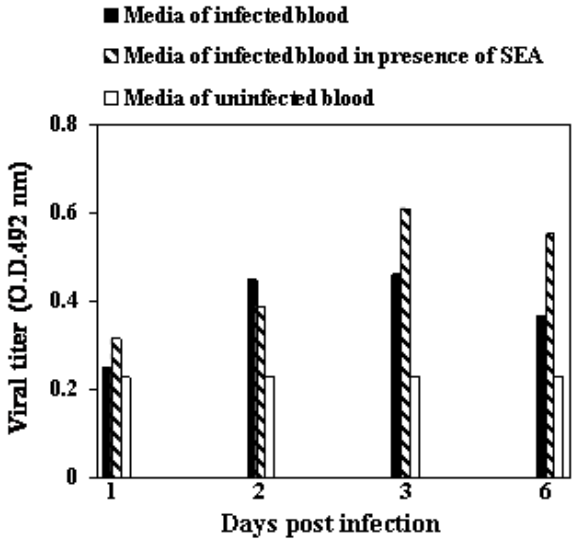
Since the HCV-E1 and E2 proteins are known to form non-covalently linked hetero dimmers [32], the 105 kDa peptide recorded by Western blot in the lysate of infected cells at week 1 and 2 post infection might be a dimmer between E1 (31-35 KDa) and E2 (70 KDa). The 76 kDa peptide might be corresponding to the viral E2 [33]. The 43 kDa peptide could be formed due to incomplete processing of the precursor polyprotein [34]. The 34 and 30 kDa peptides might be the HCV-E1 [35]. The

Figure 2. Influence of *S. mansoni* SEA existence on HCV propagation in HepG2 cells.



Using anti-HCV-core monoclonal antibody in ELISA, the recorded core titers at all time points post infection were generally higher in media of infected cells than that of control cells. Addition of *S. mansoni* SEA caused increases in the quantified core titers in the media at different time points post infection compared to media of infected cells in absence of such parasite antigens reflecting the potential of SEA to enhance viral propagation.

Figure 3. Influence of *S. mansoni* SEA existence on HCV propagation in cultured human blood cells.



Using mouse anti-HCV-protease antibodies in ELISA detected higher NS3 titers in media of infected cells compared to that of control cells. The quantified NS3 titers were higher in media of cultured blood cells in presence of SEA than in its absence, confirming the enhancing effect of such bilharzial antigens on HCV replication.

27 kDa band might represent the NS4B [36]. The 22 kDa peptide might be the HCV core [37].

RT-PCR of HCV RNA in HepG2 cells starting from day 4 post infection, agrees with previously reported detection of the virus RNA in the same cell line although using different primers and protocols [19, 21, 25].

The evident increase in the quantified core titers in the media of infected HepG2 cells upon addition of *S. mansoni* SEA reflects the potential of such parasite antigens to enhance viral propagation *in vitro*. Also, the high specificity of the used commercial monoclonal anti-core antibodies confirms successful *in vitro* infection of HepG2 cells using viremic sera, viral propagation, and shedding into the culture media. The capacity of monospecific antibodies produced against the synthetic core peptide to detect the HCV-core in infected HepG2 cells as well as precipitated immune complexes from HCV infected human sera was previously reported [21].

Also, RT-PCR results revealed earlier detection of HCV-RNA in infected cells in the presence of SEA compared to infected cells in the absence of such crude parasite antigens, yet no obvious differences were recorded in the intensities of the amplified products. The early detection of the virus genome in RNA extracted from infected cells in the presence of SEA may be interpreted in light of a previous report describing the proliferative effect of such antigens [38] that might give more space for enhancing viral replication.

Using mouse anti-HCV-protease antibodies in ELISA detected higher NS3 titers in media of human blood infected cells compared to that of control cells. This result reflects the diagnostic value of such antibodies to detect active viral infection and agrees with previous reports on susceptibility of cells from lymphatic origin to HCV infection and their support of virus replication [22-23,39-43].

The quantified NS3 titers were higher in media of cultured blood cells in the presence of SEA than in its absence, supporting the above-mentioned enhancing effect of SEA on HCV propagation observed in HepG2. Also, our results agree with previous data obtained by El-Awady *et al*. [22] upon culturing PBMCs from chronic HCV patients in the presence of homologous antigens from the parasite *S. haematobium*, although they used different primers and antibodies for detection.

The enhancing effects of SEA from *S. mansoni* (the colonic form where eggs are deposited in liver) recorded in the present work, or from *S. haematobium* (the urinary form where eggs are deposited in bladder (El-Awady *et al*. [22]), on HCV propagation draw the attention to the influence of schistosomiasis infection on the progress of the hepatitis infection and consequently the progress of liver damage irrespective to the parasite species.

Both findings open very important research questions whether individuals having HCV coinfection with *S. mansoni* or *S. haematobium* or mixed infections of both parasite species might have different extents of progress in liver damage.

Clinical studies demonstrated that antibodies against NS3 appear early in the course of HCV infection [44] before or concomitantly with sero-conversion to anti-core antibodies as the expression of NS3 is required for active viral replication. This explains the reason for including recombinant NS3 in the first [45-46], second [47] and third generation ELISA [48] for detecting anti-HCV-antibodies.

The enhancing effect of SEA for virus propagation in both HepG2 and human blood cells agrees with the frequently reported HCV/*S. mansoni* coinfection that was early thought to be due to low hygienic standards and the multiple use of needles during the early schistosomiasis control programs [10-11,49] that are no longer in existence. One might think that either infection might increase host predisposition to the other through suppressing necessary immune mechanisms induction, a process known as immune evasion.

In this regard, previous reports suggested that immune responses in chronic *S. mansoni* infections favor Th2 responses that are mounted at the expense of the Th1 responses required for clearing HCV. As a result of this skew in Th responses, susceptibility to the virus infection is enhanced [8-9, 50].

Although the *in vitro* cell culture system revealed evidence for HCV replication and HCV/*S. mansoni* antigen interaction, a susceptible animal model for both infections is still urgently needed. In this context, hope is growing in the availability of the humanized mice model that would be the best model not only to address all the immunological and pathological consequences of the coinfection but also anti-HCV drug discovery.

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References

1. Barakat R, Farghaly A, El Morshedy H, Hassan M, Miller W (1998) Impact of national schistosomiasis control program in Kafr El-Sheikh governorate, Nile Delta, Egypt: an independent evaluation. J Egypt Public Health Assoc 73: 737–753.
2. El-Sahn AA, El-Masry AG, Shehata AI, Ibrahim HF (2002) Current status of *Schistosoma mansoni* infection in El-Prince village, Alexandria Governorate. J Egypt Public Health Assoc 77: 537–552.
3. El-Ayyat AA, Sayed HA, El-Desoky HH (2003) Pattern of water contact activities in relation to *S. mansoni* infection in rural area in Giza Governorate, Egypt. J Egypt Public Health Assoc 78: 417–432.
4. Ahmed AH, Ruppel A, Ramzy RM (2003) A longitudinal study of schistosome intermediate host snail populations and their trematode infection in certain areas of Egypt. J Egypt Soc Parasitol 33: 201–217.
5. Capron A, Dessaint JP (1992) Immunologic aspects of schistosomiasis. Ann Rev Med 43: 209–218.
6. El-Gaafary MM, Rekacewicz C, Abdel-Rahman AG, Allam MF, Hosseiny ME, Hamid MA, Colombani F, Sultan Y, El-Aidy S, Fontanet A, Mohamed MK (2005) Surveillance of acute hepatitis C in Cairo, Egypt. J Med Virol 76: 520–525.
7. Lehman EM, Wilson ML (2009) Epidemic hepatitis C virus infection in Egypt: estimates of past incidence and future morbidity and mortality. J Viral Hepat 16: 650–658.
8. El-Kady IM, El-Masry SA, Badra G, Halafawy KA (2004) Different cytokine patterns in patients coinfected with hepatitis C virus and *Schistosoma mansoni*. Egypt J Immunol 1: 23–29.
9. El-Kady IM, Lotfy M, Badray G, El-Masry S, Wakedy I (2004) Interleukin (IL)-4, IL 10, IL-18 and IFN-γ cytokines pattern in patients with combined hepatitis C virus and *Schistosoma mansoni* infections. Scand J Immunol 61: 87–91.
10. Frank C, Mohamed MK, Strickland GT, Lavanchy D, Arthur RR, Magder LS, El Khoby T, Abdel-Wahab Y, Aly Ohn ES, Anwar W, Sallam I (2000) The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. Lancet 355: 887–891.
11. Habib M, Mohamed MK, Abdel-Aziz F, Magder LS, Abdel-Hamid M, Gamil F, Madkour S, Mikhail NN, Anwar W, Strickland GT, Fix AD, Sallam I (2001) Hepatitis C virus infection in a community in the Nile delta: risk factors for seropositivity. Hepatology 33: 248–253.
12. Kamal SM, Bianchi L, Al Tawil A, Koziel M, Khalifa KE, Peter T, Rasenack JW (2001) Specific cellular immune response and cytokine patterns in patients coinfected with hepatitis C virus and *Schistosoma mansoni*. J Infect Dis 184: 972–982.
13. Kamal SM, Rasenack JW, Bianchi L, Al Tawil A, Khalifa EK, Peter T, Mansour H, Ezzat W, Koziel M (2001) Acute hepatitis C without and with schistosomiasis: correlation with hepatitis C specific CD4+T-cell and cytokine response. Gastroenterology 121: 646–656.
14. Gui M, Kusel JR, Shi YE, Ruppel A (1995) Schistosoma japonicum and *Schistosoma mansoni*: comparison of larval migration patterns in mice. J Helminthol 69: 19–25.
15. Sakai A, Takikawa S, Thimme R, Meunier JC, Spangenberg HC, Govindaraj S, Farci P, Emerson SU, Chisari FV, Purcell RH, Bukh J (2007) In vivo study of the HC-TN strain of hepatitis C virus recovered from a patient with fulminant hepatitis: RNA transcripts of a molecular clone (pHC-TN) are infectious in chimpanzees but not in Huh7.5 cells. J Virol 81: 7208–7219.
16. Iacovacci S, Manzin A, Barca S, Sargiacomo M, Serafino A, Valli MB, Macioce G, Hassan HJ, Ponzetto A, Clementi M, Peschle C, Carloni G (1997) Molecular characterization and dynamics of hepatitis C virus replication in human foetal liver hepatocytes infected *in vitro*. Hepatology 26: 1328–1337.
17. Iacovacci S, Sargiacomo M, Parolini I, Ponzetto A, Peschle C, Carloni G (1993) Replication and multiplication of hepatitis C virus genome in human foetal liver cells. Res Virol 144: 275–279.
18. Ito T, Mukaigawa J, Zuo J, Hirabayashi Y, Mitamura K, Yasui K (1996) Cultivation of hepatitis C virus in primary hepatocyte culture from patients with chronic hepatitis C results in release of high titer infectious virus. J Gen Virol 77: 1043–1054.
19. Seipp S, Mueller HM, Pfaff E, Stremmel W, Theilmann L, Goeser T (1997) Establishment ofpersistent hepatitis C virus infection and replication *in vitro*. J Gen Virol 78: 2467–2476.
20. Song ZQ, Hao F, Ma QY, Wang YM (2003) *In vitro* infection of human liver cancer cell line HepG2 with HCV. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi 17: 77–80.
21. El-Awady MK, Tabll AA, El-Abd YS, Bahgat MM, Shoeb HA, Youssef SS, Bader El-Din NG, Redwan RM, El-Demellawy M, Omran MH, El-Garf WT, Goueli SA (2006) HepG2 cells support viral replication and gene expression of hepatitis C virus genotype 4 *in vitro*. World J Gastroenterol 14: 4836–4842.
22. El-Awady MK, Tabll AA, Redwan RM, Youssef S, Omran MH, Thakeb F, El-Demellawy M (2005) Flow cytometric detection of hepatitis C virus antigens in infected peripheral blood leukocytes: binding and entry. World J Gastroenterol 11: 5203–5208.
23. El-Awady MK, Youssef SS, Omran MH, Tabll AA, El Garf WT, Salem AM (2006) Soluble egg antigen of Schistosoma haematobium induces HCV replication in PBMC from patients with chronic HCV infection. BMC Infect Dis 6: 91–100.
24. Blackard JT, Smeaton L, Hiasa Y, Horiike N, Onji M, Jamieson DJ, Rodriguez I, Mayer KH, Chung RT (2005) Detection of hepatitis C virus (HCV) in serum and peripheral-blood mononuclear cells from HCV-monoinfected and HIV/HCV-coinfected persons. J Infect Dis 192: 258–265.
25. Favre D, Berthillon P, Trepo C (2001) Removal of cell bound lipoproteins: a crucial step for the efficient infection of liver cells with hepatitis C virus *in vitro*. Med Sci 324: 1141–1148.
26. Itoh Y, Okanoue T, Sakamoto S, Nishioji K, Yasui K, Sakamoto M, Kashima K (1995) Monokine production by peripheral whole blood in chronic hepatitis C patients treated with interferon. Digest Dis Sci 40: 2423–2430.
27. Mutapi M, Winborn G, Midzi N, Taylor M, Mduluza T, Maizels RM (2007) Cytokine responses to Schistosoma haematobium in a Zimbabwean population: contrasting profiles for IFN-γ, IL-4, IL-5 and IL-10 with age. BMC Infect Dis 7: 139–149.
28. Bahgat M, Sorgho H, Ouedraogo JB, Poda JN, Sawadogo L, Ruppel A (2006) Enzyme-linked immunosorbent assay with worm vomit and cercarial secretions of *Schistosoma*

mansoni to detect infections in an endemic focus of Burkina Faso. J Helminthol 80: 19–23.
29. Bahgat MM, Ibrahim AA, Abd-Elshafy DN, Mesalam AA, Gewaid HE, Ismaeil AA, El-Waseef AM, Maghraby AS, Barakat AB, El-Far MA, Ghanem HE, Mohamed AM, Ali MA (2009) Characterization of NS3 protease from an Egyptian HCV genotype 4a isolate. Arch. Virol. (Epub ahead of print).
30. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.
31. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354.
32. Op De Beeck A, Voisset C, Bartosch B, Ciczora Y, Cocquerel L, Keck Z, Foung S, Cosset FL, Dubuisson J (2004) Characterization of functional hepatitis C virus envelope glycoproteins. J Virol 78: 2994–3002.
33. Revie D, Braich RS, Bayles D, Chelyapov N, Khan R, Geer C, Reisman R, Kelley AS, Prichard JG, Salahuddin SZ (2005) Transmission of human hepatitis C virus from patients in secondary cells for long term culture. Virol J 2: 37.
34. Yasui K, Wakita T, Tsukiyama-Kohara K, Funahashi SI, Ichikawa M, Kajita T, Moradpour D, Wands JR, Kohara M (1998) The native form and maturation process of hepatitis C virus core protein. J Virol 72: 6048–6055.
35. Blanchard E, Brand D, Trassard S, Goudeau A, Roingeard P (2002) Hepatitis C virus-like particle morphogenesis. J. Virol. 76: 4073–4079.
36. Major ME, Rehermann B, Feinstone FM (2001) Hepatitis C virus. Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (editors), Fields virology, 4th edition, pp. 1127–1162.
37. Selby MJ, Choo QL, Berger K, Kuo G, Glazer F, Eckart M, Lee C, Chien D, Kuo C, Houghton M (1993) Expression, identification and subcellular localization of the proteins encoded by the hepatitis C viral genome. J Gen Virol 74: 1103–1113.
38. El-Awady MK, Gad YZ, Wen Y, Eassawi M, Effat L, Amr KS, Ismail S, Christ GJ (2001) Schistosoma haematobium soluble egg antigens induce proliferation of urothelial and endothelial cells. World J Urol. 19: 263–266.
39. Shimizu YK, Iwamoto A, Hijikata M, Purcell RH, Yoshikura H (1992) Evidence for *in vitro* replication of hepatitis C virus genome in a human T-cell line. Proc Natl Acad Sci USA 89: 5477–5481.
40. Cribier B, Schmitt C, Bingen A, Kirn A, Keller F (1995) *In vitro* infection of peripheral blood mononuclear cells by hepatitis C virus. J Gen Virol 76: 2485–2491.
41. Nakajima N, Hijikata M, Yoshikura H, Shimizu YK (1996) Characterization of long-term cultures of hepatitis C virus. J Virol 70: 3325–3329.
42. Sugiyama K, Kato N, Mizutani T, Ikeda M, Tanaka T, Shimotohno K (1997) Genetic analysis of the hepatitis C virus (HCV) genome from HCV-infected human T cells. J Gen Virol 78: 329–336.
43. Radkowski M, Bednarska A, Horban A, Stanczak J, Wilkinson J, Adair DM, Nowicki M, Rakela J, Laskus T (2004) Infection of primary human macrophage with hepatitis C virus *in vitro*: induction of tumor necrosis factor-α and interleukin 8. J Gen Virol 85: 47–59.
44. Van der Poel CL, Cuypers HT, Reesink HW, Weiner AJ, Quan S, Di Nello R, Van Boven JJ, Winkel I, Mulder-Folkerts D, Exel-Oehlers PJ (1991) Confirmation of hepatitis C virus infection by new four-antigen recombinant immunoblot assay. Lancet 337: 317–319.
45. Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE (1989) An assay for circulating antibodies to a major etiologic virus of human non-A non-B hepatitis. Science 244: 362–364.
46. Inchauspe G, Major ME, Nakano I, Vitvitski L, Trepo C (1997) DNA vaccination for the induction of immune responses against hepatitis C virus proteins. Vaccine 15: 853–856.
47. Chien DY, Choo QL, Tabrizi A, Kuo C, McFarland J, Berger K, Lee C, Shuster JR, Nguyen T, Moyer DL, Tong M, Furuta S, Omata M, Tegtmeier G, Alter H, Schiff E, Jeffers L, Houghton M, Kuo G (1992) Diagnosis of hepatitis C virus (HCV) infection using an immunodominant chimeric polyprotein to capture circulating antibodies: reevaluation of the role of HCV in liver disease. Proc Natl Acad Sci. USA 89: 10011–10015.
48. Uyttendaele S, Claeys H, Mertens W, Verhaert H, Vermylen C (1994) Evaluation of third-generation screening and confirmatory assays for HCV antibodies. Vox Sang 66: 122–129.
49. Angelico M, Renganathan E, Gandin C, Fathy M, Profili MC, Refai W, De Santis A, Nagi A, Amin G, Capocaccia L, Callea F, Rapicetta M, Badr G, Rocchi G (1997) Chronic liver disease in Alexandria governorate, Egypt: contribution of schistosomiasis and hepatitis virus infections. J Hepatol 26: 236–243.
50. Farid A, Al-Sherbiny M, Osman A, Mohamed N, Saad A, Shata MT, Lee DH, Prince AM, Strickland GT (2005) Schistosoma infection inhibits cellular immune responses to core HCV peptides. Parasite Immunol 27: 189–196.

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